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(54) Title: MUTANT CYTOKINES HAVING INCREASED RECEPTOR AFFINITY

(57) Abstract

A variant of a naturally-occurring cytokine having a Phe-Leu or Tyr-Leu sequence in a helical domain and a negatively charged amino acid within two amino acids immediately upstream or downstream from said Phe-Leu or Tyr-Leu sequence, said variant having an increased affinity for the receptor of said naturally-occurring cytokine by virtue of a neutral or basic amino acid having been substituted for a said negatively charged amino acid.

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MUTANT CYTOKINES HAVING INCREASED RECEPTOR AFFINITY

Background of the Invention

The invention relates to mutant cytokines having an altered receptor affinity.

PCT Application PCT/US86/02464 discloses native and mutant forms of interleukin-4 from a variety of mammalian sources.

Taniguchi et al. (U.S. Patent No. 4,738,927) discloses a gene encoding interleukin-2.

Murphy et al. (U.S. Patent No. 4,675,392) disclose cytotoxic fusion proteins that include a portion of interleukin-2.

Bazan (*Immunology Today* 11:350, 1990) describes structural models of cytokine receptor interactions.

Cunningham et al., (*Science* 244:1081, 1989) describe alanine substitution mutants of human growth hormone, including a substitution at Glu^{174} which increases affinity.

Summary of the Invention

The invention features variants of naturally-occurring cytokines containing a Phe-Leu or Tyr-Leu sequence in an alpha helical domain and a negatively-charged amino acid (Glu or Asp) within two amino acids immediately upstream or downstream from the Phe-Leu or Tyr-Leu sequence; the variants have an increased affinity for the receptor of the naturally-occurring cytokine by virtue of a neutral (Gly, Ala, Val, Ile, Leu, Phe, Pro, Met, Ser, Thr, Tyr, Trp, Asn, Gln, Lys) amino acid which is substituted for the negatively-charged amino acid which is within two amino acids immediately upstream or downstream of the Phe-Leu or Tyr-Leu sequence.

In general, the invention features a variant of a naturally-occurring cytokine having a Phe-Leu or Tyr-Leu

sequence in a alpha helical domain and a negatively charged amino acid within two amino acids immediately upstream or downstream from the Phe-Leu or Tyr-Leu sequence, the variant having an increased affinity for the receptor of the naturally-occurring cytokine by virtue of a neutral amino acid having been substituted for the negatively charged amino acid. Receptor affinity can be measured using standard receptor binding assays.

In preferred embodiments, the negatively charged amino acid is aspartic acid or glutamic acid; and the naturally occurring cytokine is an interleukin. In a more preferred embodiment, the interleukin is IL-4. In a even more preferred embodiment, the IL-4 is human IL-4.

In a related aspect, the invention features a hybrid molecule which includes a first and a second portion joined together covalently, the first portion includes a receptor-binding portion of the above-described variant cytokine and the second portion includes a molecule having enzymatic activity. In a preferred embodiment, the enzymatic activity is capable of decreasing cell viability. By "reduces viability" is meant kills or inhibits proliferation.

In a preferred embodiment, the second portion includes a cytotoxin. In a more preferred embodiment, the cytotoxin is a fragment of a peptide toxin which is enzymatically active but which does not possess generalized eukaryotic receptor binding activity. In an even more preferred embodiment, the fragment of a peptide toxin includes fragment A of diphtheria toxin and enough of fragment B of diphtheria toxin to form a pore in a cell membrane.

The variants of the invention generally are based on cytokines which, like human IL-4, have the sequence Phe-Leu or Tyr-Leu in an alpha helix; the alpha helix generally is within the carboxy half of the cytokine and

is usually the most carboxy-terminal alpha helix. Cytokines having the aforementioned characteristics are referred to as human IL-4 related cytokines. Examples of human IL-4 related cytokines include: human prolactin, human interleukin-2, human interleukin-6, and human interleukin-8. Generally, the alpha helices of human IL-4 related cytokines encompassing the above-described Phe-Leu (or Tyr-Leu) sequence are amphiphilic. With regard to protein structure an alpha helix is a structure in which the backbone of the peptide chain forms a helix. In such a helix there are roughly 3.6 amino acids per turn of the helix. An amphiphilic alpha helix has a charged face and an uncharged face.

A cytokine has characteristics which permit its classification as a human IL-4 related cytokine if two criteria are meet. First, there must be an Phe-Leu (or Tyr-Leu) sequence in the carboxy terminal half of the cytokine. Second, the Phe-Leu or Tyr-Leu sequence must be within an alpha helix. While extensive structural analysis, e.g., x-ray crystallography or NMR analysis, may be required to make such a determination with complete certainty, for the purposes of the identifying human IL-4 related cytokines a determination that the Phe-Leu or Tyr-Leu sequence lies within a region which has the characteristics of an alpha helix forming sequence is sufficient. Such secondary structure predictions can be made using well-known techniques (Chothia et al., *J. Biol. Chem.* 145:215, 1981; Cohen et al., *J. Mol. Biol.* 132:275, 1979; Cohen et al., *Proteins* 7:1, 1990; Cornette et al., *J. Mol. Biol.* 195:659, 1987; Bazan et al., *Proc. Natl. Acad. Sci. USA* 85:7872, 1988; Richardson et al., *Science* 240:1648, 1988). Secondary structure prediction can also be made using a computer program such as *PCGENE*™ (IntelliGenetics, Mountain View, CA). If such analysis indicates that the Phe-Leu (or

Tyr-Leu) sequence lies within an alpha helix, then the cytokine is a human IL-4 related cytokine. In general, the alpha helix containing the Phe-Leu or Tyr-Leu sequence is the most carboxy-terminal alpha helix. The finding that the alpha helix is amphipathic gives added assurance that the cytokine is a human IL-4 related cytokine, but is not required to fulfill the criteria for inclusion. Bazan (*supra*) describes structural features of cytokines.

The increased affinity cytokine variants of the invention can be used in any application currently employing the naturally occurring cytokine. Of particular importance are hybrid toxin molecules in which the cytokine or a receptor binding portion thereof replaces the generalized eukaryotic binding domain of toxins such as diphtheria toxin to form a hybrid molecule capable of selectively targeting an unwanted class of cells, e.g., IL-2 receptor- or IL-4 receptor-bearing cells involved in human disease such as T-cell lymphoma. The increased affinity of the variants of the invention for the receptor will render the hybrid molecules more toxic than hybrids made using the corresponding naturally-occurring cytokine, and thus smaller dosages will be required to achieve the same therapeutic effect.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

<u>Detailed Description</u>

The drawings are first briefly described.

Fig. 1 is a graph illustrating the effect of various molecules on binding of [^{125}I]-mIL-4 bound to P815 cells. The percentage of the maximum amount [^{125}I]-mIL-4 bound is plotted as a function of the concentration (M)

of mIL-4 (○), DAB_{389}-mIL-4(D^{495} →N) (△), DAB_{389}-mIL-4 (○), DAB_{389}-mIL-4(F^{496} →A) (♦), or DAB_{389}-mIL-4(F^{496} →P) (■).

Fig. 2 is a graph illustrating the effect of DAB_{389}-mIL-4 and DAB_{389}-mIL-4 variants on incorporation of [^{14}C]-leucine into P815 murine mastocytoma cell. Percent incorporation (relative to untreated cells) is plotted as a function of the concentration (M) of DAB_{389}-mIL-4 (○), DAB_{389}-mIL-4(D^{495} →N)(•), DAB_{389}-mIL-4(F^{496} →A) (♦), or DAB_{389}-mIL--4(F^{496} →P) (■).

<u>Cytokine Structure and Receptor Binding</u>

A number of cytokines have similar structural elements. In particular, several that are known to bind to one or another member of the hematopoietin cytokine receptor superfamily are predicted to have a carboxy terminal alpha helix. In many cases the alpha helix is substantially amphiphilic in nature. Importantly, each has a highly conserved Phe-Leu (or Tyr-Leu) sequence within the amphipathic helix. Murine and human interleukin-4 are examples of cytokines having the above-described conserved structure.

Described below are a series of experiments employing DAB_{389}-mIL-4, a murine interleukin-4 diphtheria toxin-related fusion protein. These experiments demonstrate the importance of certain structural elements to receptor binding. DAB_{389}-mIL-4 is created by replacing the generalized cell binding domain (deletion of 97 amino acids Thr^{387} to His^{485} of diphtheria toxin with murine interleukin-4 (mIL-4). Thus, the amino terminus of mIL-4 is linked by a peptide bond to a carboxy-terminal portion of diphtheria toxin. This molecule is selectively toxic to cells bearing the mIL-4 receptor. Using site directed and in-frame deletion mutagenesis to alter the mIL-4 portion of DAB_{389}-mIL-4, we have found that deletion of the carboxy-terminal 15 amino acids of mIL-4, substitution of Phe^{496} with either Pro, Ala, or Tyr, or

substitution of Leu^{497} with either Ala or Glu decreases binding to the mIL-4 receptor and cytotoxicity. In contrast, the substitution of the negatively changed residue Asp^{495} with Asn results in a four-fold increase in cytotoxic potency and binding affinity to mIL-4 receptor bearing cells *in vitro*.

<u>DAB_{389}mIL-4 Variants</u>

DAB_{389}mIL-4 is a fusion protein in which the receptor binding domain of native diphtheria toxin has been replaced by murine interleukin-4 (mIL-4). DAB_{389}mIL-4 selectively binds to eukaryotic cells expressing the murine IL-4 receptor (mIL-4R), causing them to internalize the DAB-derived portion of the molecule which inhibits protein synthesis in the cells, causing cell death. DAB_{389}mIL-4 cytotoxicity is inhibited by excess mIL-4 or antibodies directed against mIL-4, demonstrating that entry of DAB_{489}mIL-4 into target cells is mediated by the mIL-4 receptor.

We have modified the mIL-4 portion of DAB_{389}mIL-4 to create variants with altered toxicity towards mIL-4R bearing cells. Since the change is in the mIL-4 portion of the molecule, it follows that altered toxicity is caused by altered affinity of the molecule for the mIL-4 receptor. Thus, these same mutations, when introduced into a related fusion protein, should alter its affinity for its receptor in an analogous fashion.

Described below are several DAB_{389}mIL-4 variants created by site directed mutagenesis. The ability of each variant to bind and intoxicate cells bearing the mIL-4 receptor was tested. The results permit prediction of the effect of mutation on IL-4 related cytokines including human IL-2, human IL-6, human IL-8.

Several in-frame deletions and point mutations were introduced into the mIL-4 segment of the structural gene encoding DAB_{389}mIL-4 carried on plasmid pFL389

(Lakkis et al., *Eur. J. Immunol.* 21:2253, 1991). Briefly, oligonucleotide site-directed mutagenesis was performed using a T7-Gen *in vitro* mutagenesis kit (United States Biochemical, Cleveland, OH) based on the previously published work (Vandeyar et al., *Gene* 65:129, 1988). The mIL-4 cDNA obtained from plasmid pFL389 was cloned into the SphI-HindIII sites of M13(mp19) viral vector. The single stranded viral DNA served as the template in all mutagenesis reactions. Oligonucleotide primers were synthesized on an Applied Biosystems 391A DNA synthesizer. Correct base substitution was confirmed by DNA sequencing (Sanger et al., 1977). The mIL-4 cDNA fused to a truncated diphtheria toxin gene on the expression plasmid pFL389 was then substituted with the respective mutated mIL-4 cDNAs. In-frame deletion mutations were constructed by utilizing convenient restriction endonuclease sites in the fusion gene and by using oligonucleotide linkers. *E. Coli* JM101 (BRL/GIBCO, Bethesda, MD) was used throughout.

Recombinant DAB_{389}mIL-4 was prepared from recombinant *E. coli* grown in 10 liters of M9 minimal medium (1mM $MgSO_4$, 0.1mM $CaCl_2$, 0.0005% thiamine, 0.5% glycerol) supplemented with 1% Casamino acids (Difco) and 100 µg/ml ampicillin in a New Brunswick Microferm. Cultures were incubated at 30°C and sprayed with air at 10 L/min. When the absorbance (A_{590nm}) of the culture reached 0.3 - 0.5, expression of the chimeric *tox* gene was induced by the addition of 1 gm isopropyl-β-D-thiogalactopyranoside (IPTG) (United States Biochemicals, Cleveland, OH). Ninety min after induction, bacteria were harvested by centrifugation, resuspended in lysis buffer (50mM KH_2PO_4, 10mM EDTA, 750mM NaCl, 0.1% Tween 20, pH 8.0) and disrupted by sonication (Branson). The bacterial lysate was centrifuged at 2,500 x g for 20 min at 4°C to remove whole bacteria and debris, and the

clarified lysate was applied to an anti-diphtheria toxin immunoaffinity column. Bound proteins were eluted from the immunoaffinity column with elution buffer (4M guanidine hydrochloride, 100mM KH_2PO_4, 0.1% Tween 20, pH 7.2). Recombinant DAB_{389}-mIL-4 fusion toxins were further purified by high pressure liquid chromatography on a 7.5 x 600mm G4000PW column (TosoHass). Prior to use the fusion toxins were exhaustively dialyzed against phosphate buffered saline (PBS, pH 7.4). Protein concentration was determined by the Bradford method (Pierce Chemical Co., Rockford, IL).

Table 1 summarizes the single amino acid substitutions introduced into DAB_{389}mIL-4. In addition, two deletion mutants were expressed and purified: DAB_{389}-mIL-4(Δ495-509) is a variant of DAB_{389}-mIL-4 that lacks the 15 carboxy-terminal amino acids, while DAB_{389}-mIL-4(Δ390-475) consists of the first 389 amino acids of diphtheria toxin fused directly to the fifth cysteine residue in mIL-4 by a His-Ala encoding oligonucleotide linker. Analysis of DAB_{389}-mIL-4 and mutant fusion toxins on coomassie blue stained 12% SDS-polyacrylamide gels indicated that the proteins were highly purified. Their electrophoretic mobilities corresponded to their respective molecular weights as deduced from DNA sequence analysis. However, DAB_{389}-mIL-4($F^{496}\rightarrow P$), in which Phe^{496} is replaced with a Pro residue, had slightly aberrant electrophoretic mobility.

TABLE 1: DAB$_{389}$ mIL-4 Variants

PLASMID	<u>TOX</u> GENE PRODUCT
pFL389	DAB$_{389}$-mIL-4
pFL389 (15)	DAB$_{389}$-mIL-4(Δ495-509)
pFL389 (86)	DAB$_{389}$-mIL-4(Δ390-475)
pFL389(F→P)	DAB$_{389}$-mIL-4(F^{496}→P)
pFL389(F→A)	DAB$_{389}$-mIL-4(F^{496}→A)
pFL389(F→Y)	DAB$_{389}$-mIL-4(F^{496}→Y)
pFL389(L→A)	DAB$_{389}$-mIL-4(L^{497}→A)
pFL389(L→E)	DAB$_{389}$-mIL-4(L^{497}→E)
pFL389(D→N)	DAB$_{389}$-mIL-4(D^{495}→N)
pFL389(K→A)	DAB$_{389}$-mIL-4(K^{494}→A)
pFL389(K→L)	DAB$_{389}$-mIL-4(K^{489}→L)
pFL389(C→G)	DAB$_{389}$-mIL-4(C^{476}→G)

<u>*In vitro* cytotoxic activity of DAB$_{389}$-mIL-4 and related variants on mIL-4 receptor bearing cells</u>

The cytotoxic action of DAB$_{389}$-mIL-4 requires binding to the mIL-4 receptor, receptor-mediated endocytosis, passage through an acidic compartment, and delivery of the ADP-ribosyltransferase component of the fusion toxin into the cytosol (Lakkis et al., *supra*). In order to investigate the effect of mutations in the mIL-4 component of DAB$_{389}$-mIL-4 on the function of this fusion protein, we have tested the *in vitro* cytotoxic potency of DAB$_{389}$-mIL-4 and related variant fusion toxins on the P815 murine mastocytoma cell line by means of a [^{14}C]-leucine uptake assay. Briefly, P815 murine mastocytoma cells (American Type Culture Collection, Rockville, MD), maintained in RPMI 1640 medium supplemented with 10%

fetal bovine serum (Cellect, BRL/GIBCO, Bethesda, MD), 2 mM glutamine, and penicillin and streptomycin to 50 IU and 50 μg/ml, respectively. For cytotoxicity assays, cells were seeded in 96-well plates (Linbro-Flow laboratories, McLean, VA) at a concentration of 1 x 10^4 per well. Fusion toxins were added in varying concentrations and the cultures were incubated for 40 hours at 37°C in a 5% CO_2 incubator. Following incubation, the cells were pulsed with [^{14}C]-leucine and radioactivity incorporated into proteins was measured essentially as described earlier by Williams et al. (*J. Biol. Chem.* 265:11885, 1990). All assays were performed in quadruplicate. Dose response curves (Fig. 1) compare the percent incorporation of [^{14}C]-leucine by the fusion toxin treated cultures relative to untreated controls.

The results of the cytotoxicity assays are presented in Table 2, where IC_{50} is the concentration of fusion toxin required for a 50% reduction in protein synthesis. Values are means of 3-5 independent experiments. Standard deviations were within 25% of the respective means.

TABLE 2: Cytotoxicity and receptor binding affinity of DAB_{389}-mIL-4 and variants.

Fusion Toxin	IC_{50} (M)	K_i (M)
DAB_{389}-mIL-4	1×10^{-9}	2.1×10^{-7}
DAB_{389}-mIL-4(Δ495-509)	$> 1 \times 10^{-7}$	$> 1.0 \times 10^{-6}$
DAB_{389}-mIL-4(Δ390-475)	$> 1 \times 10^{-7}$	$> 1.0 \times 10^{-6}$
DAB_{389}-mIL-4($F^{496}\rightarrow P$)	$> 1 \times 10^{-7}$	$> 1.0 \times 10^{-6}$
DAB_{389}-mIL-4($F^{496}\rightarrow A$)	5×10^{-8}	$> 1.0 \times 10^{-6}$
DAB_{389}-mIL-4($F^{496}\rightarrow Y$)	3×10^{-8}	$> 1.0 \times 10^{-6}$
DAB_{389}-mIL-4($L^{497}\rightarrow A$)	3×10^{-8}	$> 1.0 \times 10^{-6}$
DAB_{389}-mIL-4($L^{497}\rightarrow E$)	$> 1 \times 10^{-7}$	$> 1.0 \times 10^{-6}$
DAB_{389}-mIL-4($D^{495}\rightarrow N$)	4×10^{-10}	5.2×10^{-8}
DAB_{389}-mIL-4($K^{494}\rightarrow A$)	1×10^{-9}	4.0×10^{-7}
DAB_{389}-mIL-4($K^{489}\rightarrow L$)	2×10^{-9}	3.8×10^{-7}
DAB_{389}-mIL-4($C^{476}\rightarrow G$)	$> 1 \times 10^{-7}$	$> 1.0 \times 10^{-6}$

Deletion of the carboxy-terminal 15 amino acids of DAB_{389}-mIL-4 results in a complete loss of cytotoxic activity in the mutant DAB_{389}-mIL-4(Δ495-509) (Table 1). This deletion encompasses residues Asp^{495}-Met^{504} and indicates that the C-terminal region of IL-4 is required for the cytotoxic activity of the fusion toxin. In order to examine whether this segment alone was capable of targeting a diphtheria toxin-based fusion protein into the cytosol of mIL-4 receptor expressing cells, we then constructed a deletion mutant of DAB_{389}-mIL-4 in which Thr^{389} was fused to Cys^{476} through an oligonucleotide linker. As shown in Table 1, DAB_{389}-mIL-4(Δ390-475) is also devoid of cytotoxic activity. These observations

suggest that other regions of the mIL-4 component of DAB_{389}-mIL-4, acting alone or in concert, are required for biological activity of the fusion toxin.

We next examined two DAB_{389}mIL-4 mutants in which Phe^{496} is replaced with either Pro or Ala. Fig. 1, in which percent incorporation of $[^{14}C]$-leucine by fusion toxin treated cultures relative to untreated controls is plotted as a function of fusion toxin concentration (M), shows that both mutations DAB_{389}-mIL-4($F^{496}{\rightarrow}P$) (filled squares) and DAB_{389}mIL-4($F^{496}{\rightarrow}A$) (filled diamonds) result in a marked reduction in cytotoxic potency to P815 cells. To further analyze the role of Phe^{496} in the cytotoxic process, the activity of DAB_{389}-mIL-4($F^{496}{\rightarrow}Y$), in which Tyr was substituted for Phe^{496}, was tested. Since tyrosine has a hydroxyl group on carbon-4 in the phenyl ring, position 496 in DAB_{389}-mIL-4($F^{496}{\rightarrow}Y$) is occupied by a more polar residue which is otherwise structurally similar to phenylalanine. As shown in Table 2, this mutation, DAB_{389}-mIL-4($F^{496}{\rightarrow}Y$), causes a 13-fold decrease in cytotoxic potency.

Since a Phe-Leu pair is almost invariably found in the carboxy terminal helices of human IL-4-related cytokines, we have also examined the role of Leu^{497} in the cytotoxic activity of DAB_{389}-mIL-4. Substitution of Leu^{497} with either an Ala or Glu [DAB_{389}-mIL-4($L^{497}{\rightarrow}A$) and DAB_{389}-mIL-4($L^{497}{\rightarrow}E$), respectively] results in a marked loss of cytotoxic potency (Table 2).

In contrast to the negative effect of the above substitutions on the biological activity of DAB_{389}-mIL-4, we have found that mutations involving some of the hydrophilic residues in the region surrounding the conserved Phe-Leu pair result in either no change or an increase in cytotoxicity. For example, substitution of Lys^{494} with Ala does not alter the cytotoxic potency of the resulting fusion toxin (Table 2). DAB_{389}-mIL-

4($D^{495}{\rightarrow}N^{495}$), in which Asp^{495} is replaced with an uncharged Asn, is approximately four-fold more cytotoxic than the parental fusion toxin, DAB_{389}-mIL-4 (Table 2). In addition, substitution of Asp^{495} with an Ala also results in four-fold increase in cytotoxic potency. It is of interest to note that Asp^{495} is adjacent to the Phe-Leu pair. Mutations which increase the relative hydrophobicity of this region of the helix have resulted in an increase in the biological activity of the fusion toxin.

Although predicted carboxy-terminal alpha helix on mIL-4 displays significant amphiphilic character, the hydrophobic face of the helix is interrupted by a charged residue, Lys^{489}. We, therefore, tested whether a Lys^{489} to a Leu mutation would enhance the amphiphilicity of this segment and possibly improve the cytotoxic potency of the fusion toxin (Table 2). Interestingly, DAB_{389}-mIL-4($Lys^{489}{\rightarrow}$Leu) demonstrates 2-3 fold less cytotoxicity than the parental DAB_{389}-mIL-4 form of the fusion toxin (Table 2). Finally, substitution of Cys^{476} with a Gly residue led to total loss of cytotoxic activity.

<u>Apparent affinities of DAB_{389}-mIL and related variant fusion toxins to the m-IL-4 receptor</u>

The biological activity of DAB_{389}-mIL-4 has been shown to be mediated through the mIL-4 receptor on target cells since cytotoxicity could be specifically blocked with either excess mIL-4 or antibody to mIL-4 (Lakkis et al., *supra*). Since we have introduced mutations into the mIL-4 component of the fusion toxin, it is likely that the observed changes in the cytotoxicity of the DAB_{389}-mIL-4 variants are due to altered binding to the mIL-4 receptor. To test this hypothesis, we conducted a series of competitive displacement experiments using [^{125}I]-labeled recombinant mIL-4. (Recombinant murine IL-4,

Sterling Drug Inc., Malvern, PA was radioiodinated using the Enzymobead reagent, BioRad, Richmond, GA).

Briefly, P815 cells were washed with tissue culture medium, resuspended in PBS (pH 7.4) containing 3 mg/ml BSA at 1 x 10^6 cells reaction tube, and incubated with 175 pM $[^{125}I]$-mIL-4 in the presence or absence of increasing concentrations of unlabeled recombinant mIL-4 (Sterling Drug Inc., Malver, PA) or DAB_{389}-mIL-4 fusion toxins for 25 minutes at 37°C under 5% CO_2. The reaction was overlaid on a mixture of 80% 550 fluid (Accumetric Inc., Elizabethtown, KN) and 20% mineral oil (Sigma, St. Louis, MO) and microcentrifuged for 2 minutes. The aqueous phase and pellet of each sample, representing free and bound ligand respectively, were then counted in a Beckman Gamma 5500 counter. Inhibition constants, K_i, were calculated from the Cheng-Prusoff equation (Cheng et al., *Biochem. Pharm.* 22:3099, 1973).

As shown in Fig. 2, where the percentage $[^{125}I]$-labeled recombinant mIL-4 bound is plotted as a function of fusion toxin concentration (M), there is a direct correlation between the cytotoxic potency of a given fusion toxin and its affinity to the mIL-4 receptor. While neither DAB_{389}-mIL-4(F^{496}→P) (filled squares) nor DAB $_{389}$-mIL-4(F^{496}→A) (filled diamonds) significantly displaced $[^{125}I]$-mIL-4 bound to P815 cells, DAB_{389}-mIL-4(D^{495}→N) (open diamonds) appears to have a four-fold higher affinity than parental DAB_{389}-mIL-4 (K_i = 5.2 x 10^{-8}M versus 2.1 x 10^{-7} respectively). Displacement of mIL-4 (filled circles) was measured as a control. The K_i and IC_{50} values for DAB_{389}-mIL-4 and each of the mutant fusion toxins are summarized in Table 2. The inhibition constants were calculated following the Cheng-Prusoff equation using 129 ± 5 pM (mean ± SD of 3 independent experiments) for the radioligand equilibrium dissociation constant. Values are means of 3-5 independent

experiments. Standard deviations were within 25% of the respective means. Taken together, the data presented in Table 1 demonstrates that mutations in the C-terminus which alter cytotoxic potency also modify the affinity of the respective fusion toxin for the mIL-4 receptor.

Structural characteristics of DAB_{389}-mIL-4 and related variant fusion toxins

In an attempt to explore the mechanism by which mutations in the carboxy-terminal segment of the mIL-4 component influence binding of the fusion toxin to the mIL-4 receptor, we have examined the electrophoretic mobilities of the parental and mutant fusion toxins under native, non-denaturing conditions. These studies demonstrated that these proteins are predominantly monomeric in non-denaturing buffer. These data imply that the loss in cytotoxic activity and binding affinity observed in DAB_{389}-mIL-4 variants is not due to increased aggregation of the respective fusion toxins. We then investigated the possibility that marked reduction in affinity could be due to altered tertiary structure of the mIL-4 component of the fusion toxin. Non-denatured fusion proteins were therefore transferred to nitrocellulose paper and immunoblotted (Towbin et al., *Proc. Natl. Acad. Sci. USA*, 76:4350, 1979) with a conformationally sensitive monoclonal antibody to mIL-4 (11B11, Genzyme, Boston, MA). DAB_{389}-mIL-4 is immunoblot positive when probed with 11B11 only following electrophoresis under non-denaturing conditions (Lakkis et al., *supra*). This analyzing demonstrated that DAB_{389}-mIL-4, as well as those variants with similar or higher cytotoxic activity and binding affinity, are strongly immunoreactive with 11B11. Conversely DAB_{389}-mIL-4(Δ495-509), DAB_{389}-mIL-4(Δ390-475), DAB_{389}-mIL-4(C^{476}→G), DAB_{389}-mIL-4(F^{496}→P) and DAB_{389}-mIL-4(L_{497}→E) which are non-toxic and fail to bind to the mIL-4 receptor are not recognized

by the anti-mIL-4 monoclonal antibody. Conservative substitutions of Phe^{496} or Leu^{497}, on the other hand, are immunoblot positive but with lower intensity than DAB_{389}-mIL-4. DAB_{389}-mIL-4(L_{497}→A), for example, is 13-fold less cytotoxic than DAB_{389}-mIL-4, has a markedly reduced affinity for the mIL-4 receptor yet is significantly reactive with 11B11.

<u>Other Variant Human Growth Hormone-Related Cytokines</u>

Human IL-4-related cytokines include human IL-6, human IL-8, and human IL-2. In each case the predicted amino acid sequence indicates the presence of an acidic (negatively charged) amino acid one or two amino acids upstream of a Phe-Leu (or Tyr-Leu) sequence within the carboxy-terminal portion of the molecule. Residues which could be changed to a neutral residue include position 126 of hIL-4 (U.S. Patent 5,017,691); 117 of hIL-6 (EPA 0 261 625); the E within the sequence RVVEKFLKRA of hIL-8 (Matsushima et al., *J. Exp. Med.* 167:1883, 1988); and 136 of hIL-2 (Taniguichi et al., *Nature* 302:305, 1987). These negatively charged residues may be replaced by any neutral residue as described above for IL-4. The affinity of each variant may then be tested using standard binding assays. <u>Hybrid Molecules</u>

A variant cytokine of the invention can be linked to other molecules (e.g., a molecule with enzymatic activity) to create hybrid molecules. These hybrid molecules will bind to cells bearing the relevent cytokine receptor. This provides a convenient method for targeting molecules to cytokine receptor bearing cells. Because the variant cytokines of the invention bind their receptors with higher affinity than the related naturally occurring cytokines, the hybrid molecules will be particularly useful for directing molecules cells bearing the relevent cytokine receptor.

Hybrid cytotoxins are a useful class of hybrid molecule which can be created using the variant cytokines of the invention. Such molecules can be used to reduce the viability of cells bearing particular cytokine receptors. In these molecules a cytotoxin is linked to the variant cytokine. Hybrid cytotoxins are described by Murphy et al. (U.S. Patent 4,675,392). Other hybrid cytotoxins are described by Murphy (PCT/US90/07619). Methods for constructing such cytotoxins are well known (Murphy et al., U.S. Patent 4,675,392; Murphy, (PCT/US90/07619); Lakkis et al., *supra;* Williams et al., *J. Biol. Chem.* 265:20673, 1990; Williams et al., *J. Biol. Chem.* 265:11885, 1990). Toxicity can be tested using standard assays.

Other useful hybrid molecules are those in which a non-cytotoxic, enzymatically active molecule is linked to a variant cytokine of the invention. Such molecules, when taken up by the targeted cell, can be used to correct an enzyme deficiency or generate other molecules within the cell by enzymatic activity. It is also possible to link a detectable label to the variant cytokines of the invention for the purpose of detectably labeling receptor-bearing cells.

<u>Use</u>

The variant cytokines of the invention can be used for the same therapeutic purposes of the related naturally occurring molecule. In addition, truncated variant cytokines which bind receptor but do not elicit the normal biological response can be used to inhibit action of the naturally occurring cytokine. Because of their higher receptor affinities, such molecules employing the variant cytokines of the invention will generally be more potent than molecules employing the related naturally occurring cytokine.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The University Hospital

(ii) TITLE OF INVENTION: MUTANT CYTOKINES HAVING INCREASED RECEPTOR AFFINITY

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Fish & Richardson

(B) STREET: 225 Franklin Street

(C) CITY: Boston

(D) STATE: Massachusetts

(E) COUNTRY: U.S.A.

(F) ZIP: 02110-2804

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb

(B) COMPUTER: IBM PS/2 Model 50Z or 55SX

(C) OPERATING SYSTEM: MS-DOS (Version 5.0)

(D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 07/870,500

(B) FILING DATE: April 17, 1992

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Paul T. Clark

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(C) REFERENCE/DOCKET NUMBER: 00563/053001

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (617) 542-5070

(B) TELEFAX: (617) 542-8906

(C) TELEX: 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 576
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTAGCATCTG TTGATAAACT TAATTGTCTC TCGTCACTGA CGCACAGAGC TATTG 55

AAC CCC CAG CTA GTT GTC ATC CTG CTC TTC TTT CTC GAA TGT ACC AGG 103
Asn Pro Gln Leu Val Val Ile Leu Leu Phe Phe Leu Glu Cys Thr Arg
1 5 10 15

AGC CAT ATC CAC GGA TGC GAC AAA AAT CAC TTG AGA GAG ATC ATC GGC 151
Ser His Ile His Gly Cys Asp Lys Asn His Leu Arg Glu Ile Ile Gly
20 25 30

ATT TTG AAC GAG GTG ACA GGA GAA GGG ACG CCA TGC ACG GAG ATG GAT 199
Ile Leu Asn Glu Val Thr Gly Glu Gly Thr Pro Cys Thr Glu Met Asp
35 40 45

GTG CCA AAC GTC CTC ACA GCA ACG AAG AAC ACC ACA GAG AGT GAG CTC 247
Val Pro Asn Val Leu Thr Ala Thr Lys Asn Thr Thr Glu Ser Glu Leu
50 55 60

GTG TGT AGG GCT TCC AAG GTG CTT CGT ATA TTT TAT TTA AAA CAT GGG 295
Val Cys Arg Ala Ser Lys Val Leu Arg Ile Phe Tyr Leu Lys His Gly
65 70 75 80

AAA ACT CCA TGC TTG AAG AAG AAC TCT AGT GTT CTC ATG GAG CTG CAG 343
Lys Thr Pro Cys Leu Lys Lys Asn Ser Ser Val Leu Met Glu Leu Gln
85 90 95

AGA CTC TTT CGG GCT TTT CGA TGC CTG GAT TCA TCG ATA AGC TGC ACC 391
Arg Leu Phe Arg Ala Phe Arg Cys Leu Asp Ser Ser Ile Ser Cys Thr
100 105 110

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ATG AAT GAG TCC AAG TCC ACA TCA CTG AAA GAC TTC CTG GAA AGC CTA   439
Met Asn Glu Ser Lys Ser Thr Ser Leu Lys Asp Phe Leu Glu Ser Leu
        115                 120                 125

AAG AGC ATC ATG CAA ATG GAT TAC TCG                               466
Lys Ser Ile Met Gln Met Asp Tyr Ser
    130                 135

TAGTACTGAG CCACCATGCT TTAACTTATG AATTTTTAAT GGTTTTATTT TTAATATTTA 526

TATATTTATA ATTGATAAAA TAAAATATTT GTATAATGTA ACAGAAAAAA            576
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Claims

1. A variant of a naturally-occurring cytokine having a Phe-Leu or Tyr-Leu sequence in a alpha helical domain and a negatively charged amino acid within two amino acids immediately upstream or downstream from said Phe-Leu or Tyr-Leu sequence, said variant having an increased affinity for the receptor of said naturally-occurring cytokine by virtue of a neutral amino acid having been substituted for a said negatively charged amino acid.

2. The variant cytokine of claim 1 wherein a said negatively charged amino acid is aspartic acid or glutamic acid.

3. A hybrid molecule comprising a first and a second portion joined together covalently, said first portion comprising a receptor-binding portion of the variant cytokine of claim 1 and said second portion comprising a molecule having enzymatic activity.

4. The hybrid molecule of claim 3 wherein said molecule decreases cell viability.

5. The hybrid molecule of claim 4 wherein said second portion comprises a cytotoxin.

6. The hybrid molecule of claim 5 wherein said cytotoxin is a fragment of a peptide toxin which is enzymatically active but which does not possess generalized eukaryotic receptor binding activity.

7. The hybrid molecule of claim 6 wherein said fragment of a peptide toxin comprises fragment A of

diphtheria toxin and enough of fragment B of diphtheria toxin to form a pore in a cell membrane.

8. The variant of claim 1 wherein said naturally occurring cytokine is an interleukin.

9. The variant of claim 8 wherein said interleukin is IL-4.

10. The variant of claim 9 wherein said IL-4 is human IL-4.

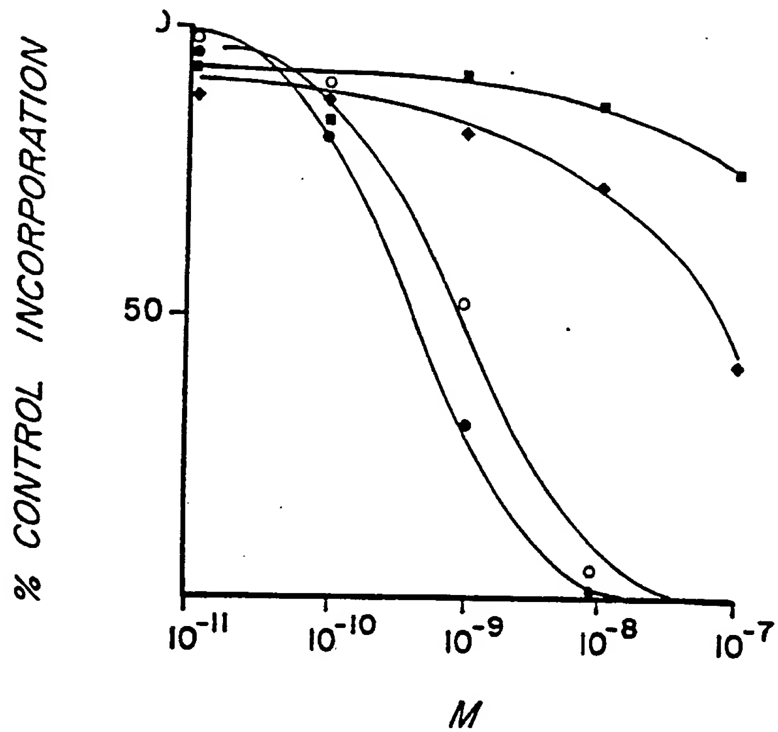

Fig. 1

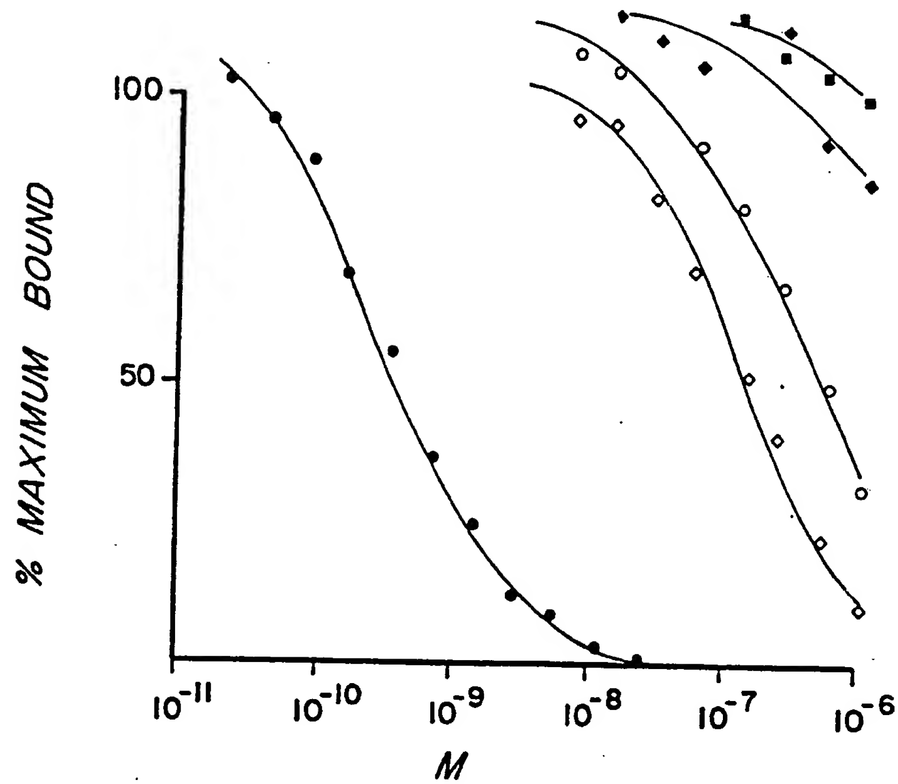

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/03613

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 13/00, 9/00; A61K 37/36
US CL :530/351; 514/2, 8
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/351; 514/2, 8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Protein Engineering, Volume 5, Number 3, issued April 1992, F. Lakkis et al., "Phe496 and Leu497 are essential for receptor binding and cytotoxic action of the murine interleukin-4 receptor targeted fusion toxin DAB389-mIL4", pages 241-248, especially the abstract, Tables I and II, and Figure 1.	3-7
A	Immunology Today, Volume 11, Number 10, issued October 1990, J. F. Bazan, "Haemopoietic receptors and helical cytokines", pages 350-354.	1-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 July 1993	14 JUL 1993

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	DAVID L. FITZGERALD
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/03613

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Proteins: Structure, Function, and Genetics, Volume 12, issued January 1992, K. Kaushansky, "Structure-Function Relationships of the Hematopoietic Growth Factors", pages 1-9.	1-10
A	Proteins: Structure, Function, and Genetics, Volume 11, issued 1991, B. M. Curtis et al., "Experimental and Theoretical Studies of the Three-Dimensional Structure of Human Interleukin-4", pages 111-119.	1-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/03613

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DATABASES: USPTO-APS; Dialog ("OneSearch" of all Biochem., Biotech, and Patent databases)
SEARCH TERMS: (Interleukin/IL)-4; mutein; site-directed; mutagen?